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Developing Salinity-Tolerant Hybrid Rice Parental Lines through Integrative Breeding and Molecular Approaches

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ABSTRACT

Salinity stress is a major abiotic constraint limiting rice production in coastal and irrigated ecosystems, affecting approximately 20% of global agricultural land. Developing salinity-tolerant hybrid rice parental lines is critical for sustaining productivity in salt-affected areas, yet systematic efforts integrating tolerance traits with fertility restoration or maintenance ability remain limited. This study reports the development of 49 new salinity-tolerant hybrid rice parental lines through strategic crossing, Best Linear Unbiased Prediction (BLUP)-based selection, and integrated molecular–phenotypic validation. A total of 286 genetically fixed entries derived from 22 crosses among the parental lines (14 maintainer × maintainer and 8 restorer × restorer), along with 19 elite lines, were evaluated under salinity stress environment. Parental lines with Standard Evaluation Score (SES) values of 3–5 were strategically crossed and advanced through field rapid generation advance (FRGA). BLUP-based selection identified 54 superior genotypes exceeding the population mean plus one standard deviation for yield. Salinity tolerance was further confirmed through screening under 12 dS/m stress at the seedling stage, which identified 10 highly tolerant parental lines (SES 3) and 19 moderately tolerant parental lines (SES 5). Molecular marker analysis using DRRM-Rf3-5 and DRCG-RF4-14 markers, coupled with test cross pollen fertility testing, successfully validated 25 maintainer lines and 24 restorer lines. Ward's D^2 hierarchical clustering classified the selected lines into three distinct clusters representing late-maturing high-yielding, medium-duration tall, and early-maturing high-tillering ideotypes. These newly developed parental lines provide valuable genetic resources for breeding high-yielding, salinity-tolerant hybrid rice varieties suited to salt-affected ecosystems, thereby contributing to sustainable rice production and enhanced food security.

Keywords: salinity tolerance, hybrid rice, parental line development, molecular markers, fertility restoration

INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food for over half the global population, and hybrid rice technology has emerged as a key strategy for enhancing productivity, delivering 15–30% yield advantages over conventional varieties through heterosis exploitation (Rout *et al.*, 2020). However, salinity stress poses a major constraint to rice production, particularly in coastal and irrigated ecosystems where approximately 20% of agricultural lands are affected (Korres *et al.*, 2022). Salinity reduces rice yield through osmotic stress and ionic toxicity, with susceptible varieties experiencing 50–70% yield losses under moderate to severe stress conditions

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(Sellathdurai *et al.*, 2024). In regions such as South Asia, Southeast Asia, and Africa, expanding salt-affected areas threaten food security and farmer livelihoods, necessitating the development of salinity-tolerant rice varieties, including hybrid rice (Mheni *et al.*, 2024).

Molecular breeding has revolutionized salinity tolerance improvement through the identification and deployment of major quantitative trait loci (QTLs), particularly Saltol on chromosome 1, which harbors the *OsHKT1;5* gene responsible for Na^+ exclusion (Rekha *et al.*, 2022). Marker-assisted backcrossing (MABC) programs have successfully introgressed *Saltol* into elite varieties, yielding significant improvements. For instance, DRR Dhan 58, developed through *Saltol* introgression into Improved Samba Mahsuri, demonstrated a 24% yield advantage in coastal saline areas and was released for cultivation (Rekha *et al.*, 2022). Similarly, the early-maturing variety KKL(R)3 showed robust seedling tolerance and substantial yield gains under saline conditions (Saminadane *et al.*, 2024). Recent meta-QTL analyses have identified 65 consensus genomic regions for salinity tolerance, providing refined targets for marker-assisted selection (Satasiya *et al.*, 2024). Salt tolerance in hybrid rice is governed by complex genetic mechanisms controlling ion homeostasis, particularly Na^+ exclusion and K^+ retention (Thippani, 2017). The major QTL *Saltol* (from Pokkali) on chromosome 1 and the *SKC1* (from Nonabokra) gene regulate Na^+/K^+ balance at seedling and reproductive stages (Yadav *et al.*, 2018). Marker-assisted selection (MAS) employs marker-assisted backcrossing with foreground and background selection using SSR, SNP, and KASP assays to introgress these loci into elite parental lines (Islam *et al.*, 2024). Donor genotypes like FL478, Pokkali, and Nona Bokra provide favorable alleles for lower shoot Na^+ and higher K^+ accumulation (Thippani, 2017). The breeding strategy involves pyramiding multiple tolerance QTLs into cytoplasmic male sterile (CMS), maintainer (B-line) and restorer (R) lines, followed by hybrid development (Niu *et al.*, 2025). Shenyanyou 1 represents a successfully developed salt-tolerant hybrid japonica rice through *SKC1* introgression using KASP markers and high-throughput breeding chips (Niu *et al.*, 2025).

Despite these advances in salinity tolerance breeding, developing hybrid rice parental lines that simultaneously possess salinity tolerance and fertility restoration or maintenance ability remains underexplored. Hybrid rice production depends on well-characterized CMS lines, B-lines, and R-lines carrying *Rf3* and *Rf4* genes (Ponnuswamy *et al.*, 2020). While marker-assisted conversion of partial restorers into complete restorers has been demonstrated (Ponnuswamy *et al.*, 2020), the integration of salinity tolerance QTLs with fertility genes in hybrid parental lines has not been systematically addressed.

In this context, the present study reports the development of 49 new salinity-tolerant hybrid rice parental lines comprising 25 B-lines and 24 R-lines. These lines were developed through strategic crossing of tolerant parents, Best Linear Unbiased Prediction (BLUP)-based selection for yield and agronomic traits, molecular marker validation of *Rf3* and *Rf4* genes, and phenotypic confirmation through pollen fertility testing. These selected parental lines were further characterized using Ward's D^2 clustering and genetic diversity analysis. This integrated breeding approach addresses a critical gap in hybrid rice breeding by providing diverse, salinity-tolerant parental lines specifically designed for developing high-yielding hybrids suitable for salt-affected ecologies, thereby contributing to sustainable rice production and global food security.

MATERIALS AND METHODS

Fourteen B×B cross and eight R×R crosses (Table 1) were done in Aman 2022-23 season following the results of seedling stage screening of 276 parental lines of hybrid rice at the net house of the Plant Physiology division, BRRI. Nineteen elite lines were collected from the salinity breeding team, Plant Breeding division, BRRI, to identify potential restorer and maintainer lines for hybrid rice development.

Table 1. B×B and R×R crosses targeting parental line development with high yield and salinity tolerance.

Cross registration no.	Cross	Female Parent SES Env 1	Female Parent SES Env 2	Male Parent SES Env 1	Male Parent SES Env 2	F_6 Entry evaluated
B×B cross						
HRB 501	BHR95/BHR182	5	5	5	5	12
HRB 502	BHR101/BHR184	5	5	5	4	33
HRB 503	BHR121/BHR187	5	5	3	4	4
HRB 510	BHR15/BHR5	5	5	8	8	13
HRB 511	BHR5/BHR15	8	8	5	5	23
HRB 512	BHR11/BHR22	4	5	3	3	14
HRB 513	BHR22/BHR11	3	3	4	5	31
HRB 516	BHR22/BHR3	3	3	8	8	10
HRB 517	BHR13/BHR8	7	7	5	5	4
HRB 518	BHR8/BHR13	5	5	7	7	5
BBC11	BHR193/BHR164	4	4	5	5	4
BBC12	BHR164/BHR193	5	5	4	4	12
BBC13	BHR182/BHR171	5	5	8	7	8
BBC14	BHR159/BHR182	5	5	5	5	9
R×R cross						
HRR 264	BHR202/BHR365	5	3	4	5	10
HRR 269	BHR323/BHR369	3	5	3	3	8
HRR 271	BHR321/BHR373	5	5	3	5	31
HRR 272	BHR323/BHR373	3	5	3	5	14
RRC3	BHR355/BHR285	3	3	5	5	3
RRC9	EL 224R /BHR371			5	5	12
RRC11	BHR334/ CHA15R	5	5			1
HRR 281	BHR321/BHR374	5	5	6	5	6
Elite lines from Plant Breeding Division, BRRI						19
Total genotypes evaluated						286

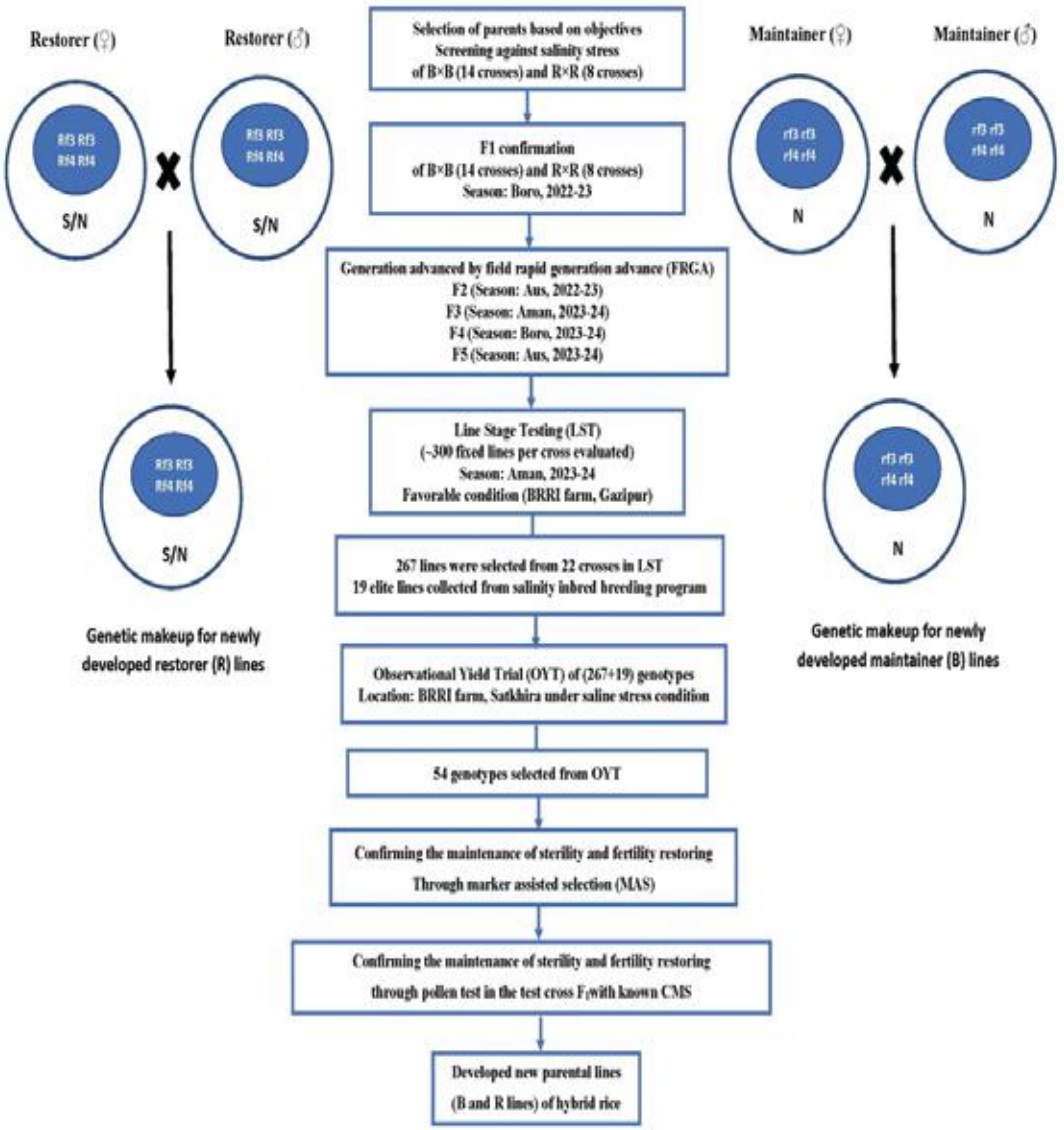
Thirty parental lines (7 BRRI-developed and 23 exotic) were used for crossing and the development of new parental lines. Among these 13 parents contain genes for salinity tolerance (e.g. *Saltol, qSIS1L*) and 7 parents contain genes for both salinity and drought tolerance, 11 parents contain genes for drought tolerance (e.g. *qDTY12.1, qDTY3.1, qDTY3.2*). Parents BHR22 and BHR365 contained both *Saltol* and *qDTY12.1, qDTY3.1, qDTY3.2* genes, conferring a dual tolerance mechanism (Table 2).

Table 2. Genes conferring salinity tolerance in hybrid rice parents utilized for new cross derived B and R line development.

Sl	HRD accession	Genotyping code	Salinity tolerance			Drought tolerance		
			Gene	Allele source	SNP marker	Gene	Allele source	SNP marker
1	BHR5	GS_5_IR77803B_1				*qDTY3.2*	IR64	IRRI_SNP1016_DTY3-2-IR64_1 (chr3: 1271431)
2	BHR8	GS_8_IR75595_B_1	*Saltol*	FL478, IR 107321-1-141-3-120	IRRI_SNP0994_SALTOL-AUS (chr1: 11460344)	*qDTY3.2*	IR64	IRRI_SNP1016_DTY3-2-IR64_1 (chr3: 1271431)
3	BHR11	GS_11_IR78361B_1	*Saltol*	Pokkali, Capsule	IRRI_SNP0995_SALTOL-ARO (chr1: 11462124)			
4	BHR13	GS_13_IR77811B_1				*qDTY12.1*	Way Rarem, IR64	IRRI_SNP1081_DTY12-1_2 (chr12: 17396363)
						qDTY3.2	IR64	IRRI_SNP1016_DTY3-2-IR64_1 (chr3: 1271431)
5	BHR22	GS_22_IR79155B_1	*Saltol*	FL478, IR 107321-1-141-3-120	IRRI_SNP0994_SALTOL-AUS (chr1: 11460344)	*qDTY12.1*	Way Rarem, IR64	IRRI_SNP1081_DTY12-1_2 (chr12: 17396363)
						qDTY3.1	Apo	IRRI_SNP1021_DTY3-1_2 (chr3: 31008659)
						qDTY3.2	IR64	IRRI_SNP1016_DTY3-2-IR64_1 (chr3: 1271431)
6	BHR101	GS_94_BRRI13A_B_1	*qSIS1L*	FL478, Capsule	IRRI_SNP1007_QSES1-2_3 (chr1: 40362958)			
7	BHR159	GS_138_BRRI100B_1	*Saltol*	Pokkali, Capsule	IRRI_SNP0995_SALTOL-ARO (chr1: 11462124)	*qDTY12.1*	Way Rarem, IR64	IRRI_SNP1081_DTY12-1_2 (chr12: 17396363)
8	BHR182	GS_157_IR105687B_1	*Saltol*	Pokkali, Capsule	IRRI_SNP0995_SALTOL-ARO (chr1: 11462124)	*qDTY3.2*	IR64	IRRI_SNP1016_DTY3-2-IR64_1 (chr3: 1271431)
9	BHR193	GS_168_IR78369B_1	*Saltol*	Pokkali, Capsule	IRRI_SNP0995_SALTOL-ARO (chr1: 11462124)			
10	BHR202	GS_172_BRRI10R_1	*Saltol*	FL478, IR 107321-1-141-3-120	IRRI_SNP0994_SALTOL-AUS (chr1: 11460344)			
11	BHR285	GS_243_BasmatiR_1	*Saltol*	Pokkali, Capsule	IRRI_SNP0995_SALTOL-ARO (chr1: 11462124)			

Sl	HRD accession	Genotyping code	Salinity tolerance			Drought tolerance		
			Gene	Allele source	SNP marker	Gene	Allele source	SNP marker
12	BHR323	GS_262_CHH_67R_1				*qDTY12.1*	Way Rarem,	IRRI_SNP1081_DTY12-1_2(chr12: 17396363)
13	BHR334	GS_273_WinR_1	*Saltol*	Pokkali, Capsule	IRRI_SNP0995_SALTOL-ARO (chr1: 11462124)			
14	BHR355	GS_294_SyngentaR_N_1_1	*Saltol*	Pokkali, Capsule	IRRI_SNP0995_SALTOL-ARO (chr1: 11462124)	*qDTY12.1*	Way Rarem, IR64	IRRI_SNP1081_DTY12-1_2(chr12: 17396363)
						qDTY3.1	Apo	IRRI_SNP1021_DTY3-1_2 (chr3: 31008659)
15	BHR365	GS_304_IR76902_C2_1	*Saltol*	Pokkali, Capsule	IRRI_SNP0995_SALTOL-ARO (chr1: 11462124)	*qDTY12.1*	Way Rarem, IR64	IRRI_SNP1081_DTY12-1_2(chr12: 17396363)
						qDTY3.1	Apo	IRRI_SNP1021_DTY3-1_2 (chr3: 31008659)
						qDTY3.2	IR64	IRRI_SNP1016_DTY3-2-IR64_1 (chr3: 1271431)
16	BHR371	GS_310_IR86522_12_1				*qDTY12.1*	Way Rarem, IR64	IRRI_SNP1081_DTY12-1_2(chr12: 17396363)
17	BHR374	GS_313_IR90928_15_1	*Saltol*	FL478, IR 107321-1-141-3-120	IRRI_SNP0994_SALTOL-AUS (chr1: 11460344)	*qDTY12.1*	Way Rarem, IR64	IRRI_SNP1081_DTY12-1_2(chr12: 17396363)

The flow chart (Fig. 1) illustrates the sequential process of hybrid rice parental line development.



Blue circle indicates nucleus, white space outside the nucleus indicates cytoplasm, Rf3 and Rf4 denote restorer alleles inside nucleus, rf3 and rf4 designate non-restorer i.e., maintainer alleles inside nucleus, S indicates the presence, and N shows the absence of male sterility gene in the cytoplasm.

Fig. 1. Sequential process of hybrid rice parental line development.

The breeding program began with the selection of parents based on breeding objectives, followed by F_1 confirmation of 14 B×B and 8 R×R crosses during Boro 2022–23. Subsequently, F_2 populations from 22 crosses were advanced through the FRGA method in Aus 2022–23, and continued progression through F_3 (Aman 2023–24), F_4 (Boro 2023–24), and F_5 (Aus 2023–24) generations following Rahman et al. (2019) with little modification of collecting and sowing first matured 5-10 seeds per plant that allowed 3 generations advancement per year.

The F_6 generation (LST) was evaluated under favorable conditions at the BRRI Farm, Gazipur during Aman 2023–24. From this evaluation, 267 selected lines, along with 19 elite collected lines, were evaluated in the Observation Yield Trial (OYT) at the BRRI Farm, Satkhira, under salinity stress conditions. Molecular confirmation was carried out to detect the restorer of fertility genes (*Rf3* and *Rf4*) using Marker-Assisted Selection (MAS), followed by pollen fertility tests in test cross F_1 plants with known CMS lines to verify sterility maintenance and fertility restoration. This systematic breeding pipeline ultimately led to the development of new B and R parental lines for hybrid rice improvement.

Salt Stress Application and Monitoring

Salt (NaCl) stress was imposed on 54 selected genotypes along with two checks at the three-leaf stage (14 days after sowing, DAS) by gradually increasing the culture solution's electrical conductivity (EC) of the nutrient solution over a six-day period to a final level of 12 dS/m. The EC was increased stepwise from 6 dS/m to 8 dS/m to 10 dS/m before reaching the final concentration. Iron (Fe) deficiency was prevented by daily adjusting the pH to 5.0 and replacing the entire Yoshida culture solution (Yoshida *et al.*, 1976) every seven days. Five seedlings per genotype were used for SES scoring. After 18 days of exposure to 12 dS/m salinity, the susceptible checks (IRRI 154 and BRRI dhan28) exhibited significant salt stress, with high SES scores of 7 to 9, which validated the severity of the stress condition and prompted the subsequent collection of SES data across all 54 genotypes.

Marker-Assisted Selection

Marker-assisted selection (MAS) was conducted using the DRRM-Rf3-5 marker located on chromosome 1 and the DRCG-RF4-14 marker located on chromosome 10 (Suresh et al., 2012; Ramalingam *et al.*, 2017) to identify potential restorer and maintainer lines of hybrid rice. PCR products were resolved on a 2% agarose gel, and gel images were documented for analysis.

Table 3. Marker details for tracking *Rf3* and *Rf4* genes

Marker	Sequences (5'-3')		Expected PCR amplicon size (bp)	Restorer	Non-restorer
	Forward Primer	Reverse Primer			
DRRM-Rf3-5	GATGGCACAGCTTCAGAACA	CTAATTCTGGGCGAGCAAAG	140/160	160	140
DRCG-RF4-14	GCAATGCTTGTATTCAGCAAA	TCCAGCTGTAAATCCGTCAA	800/885	800	885

Pollen Fertility Assessment

Fifty-four selected genotypes were crossed with the known WA-CMS (Wild Abortive Cytoplasmic Male Sterile) line BRRI97A. The resulting F_1 seeds were harvested and grown in the subsequent season. Pollen grains from the F_1 plants were gently squeezed onto a glass slide and stained with iodine-potassium iodide (I-KI) solution. Pollen exhibiting a deep stain and round morphology was classified as fertile. Observations were made using a ZEISS Axioscope 5 light microscope at 40X magnification.

Data Analysis

Data collected from Satkhira, Bangladesh (a recognized salinity hotspot) were analyzed using an augmented randomized complete block

(RCB) design. The experimental layout incorporated two check varieties, each replicated seven times. Single environment analysis was performed using Plant Breeding Tools software (PBTools, version 1.4, 2014). Best Linear Unbiased Predictors (BLUPs) and broad sense heritability estimates were calculated for each trait and utilized in selecting superior genotypes. Genetic distance estimation and cluster analysis were conducted using Ward D^2 method. DNA band scoring was carried out utilizing AlphaEaseFC 4.0 software (USA).

RESULTS

The study analyzed the development of new hybrid rice parents focusing on salinity tolerance (measured by the SES score, where low score means tolerant) across 22 distinct crosses among the parental lines of hybrid rice and 19 elite lines, resulting in a total of 286 genetically fixed entries and were evaluated. The crosses were grouped into B×B (14 crosses) and R×R (8 crosses). The parental lines exhibited varying degrees of tolerance, ranging from highly tolerant (SES 3) to highly susceptible (SES 8) across two environments (Env 1 and Env 2). For instance, the B×B cross HRB 510 (BHR15/BHR5) paired a relatively tolerant female (SES 5/5) with a highly susceptible male (SES 8/8), while HRB 502 (BHR101/BHR184) involved two tolerant parents (Female SES 5/5, Male SES 5/4). Among all crosses, HRB 502 produced the largest population for selection, with 33 F_6 entries evaluated, followed by the R×R cross HRR 271 (BHR321/BHR373) with 31 F_6 entries.

Out of the 22 crosses among the parental lines, 15 crosses were identified where both parental lines exhibited SES score of 3 to 5 in all environments, signifying that they are the most promising crosses for introgressing high salinity tolerance into new parents. These 15 crosses, comprising 9 B×B crosses and 6 R×R crosses, collectively contributed 250 F_6 entries—a substantial portion of the total evaluated population.

B×B Crosses (9 crosses)

B×B Crosses provided the highest number of individual tolerant progenies. Key examples include HRB 502 (BHR101/BHR184), which had excellent parental SES scores (Female 5/5, Male 5/4) and produced the largest single population of 33 F_6 entries among all F_6 entries, making it a critical source for high-tolerance selections. Other high-contributing crosses like HRB 501 (BHR101/BHR10) also had consistently low SES scores (5/5 for both parents) and contributed 23 entries.

R×R Crosses (6 crosses)

Although fewer in number, R×R Crosses also demonstrated high salinity tolerance. For example, HRR 264 (RRC1/RRC10) showed favorable parental SES scores (5/3 and 4/5) and contributed to 20 F_6 entries, while HRR 270 (RRC11/RRC10) contributed to 23 F_6 entries. The remaining seven crosses involved at least one parent with a highly susceptible SES score of 7 or 8, confirming that the 15 identified crosses are the primary source for the desired trait combination of high yield and high salinity tolerance in the development of new hybrid rice parental lines.

Genotype selection

A total of 286 genotypes were evaluated using an augmented Randomized Complete Block (RCB) design with two checks replicated seven times. The salinity condition of the experimental site ranged from 4.2-7.3 dSm^{-1} (Fig. 2).

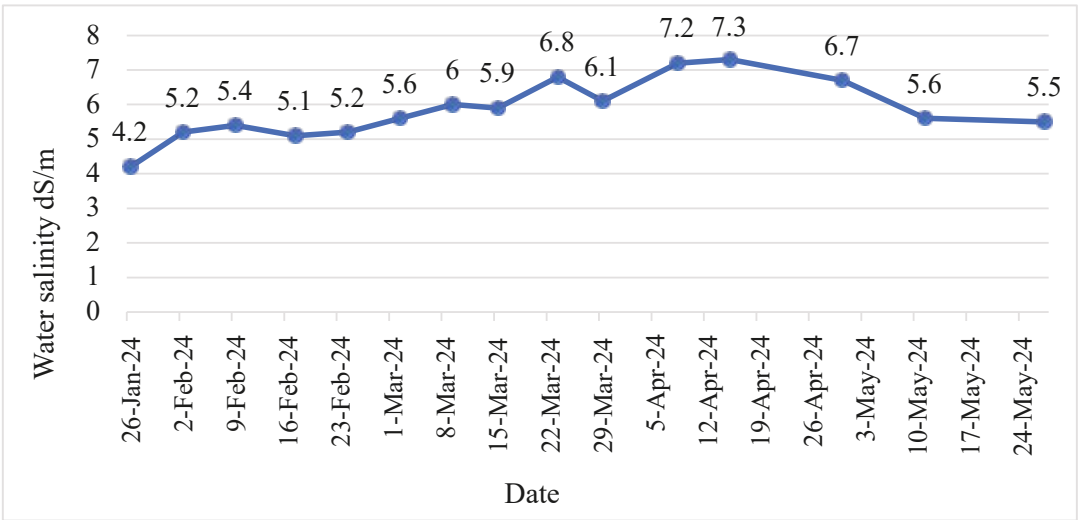


Fig. 2. Salinity stress at the experimental plot in BRRI regional station, Satkhira farm during Boro, 2023-24.

Selection was based on Best Linear Unbiased Predictor (BLUP) values for yield (kg/1.2m2), growth duration (days), plant height (cm), effective tiller number (ET) (Table 4). Genotypes were selected if their yield exceeded both the checks and the population mean plus one standard deviation (μ + 1σ). Only five specific genotypes—BR11716-4R-105, BR11716-4R-120, BR11712-4R-227, BR11715-4R-186, and BR11712-4R-1 achieved a yield greater than the population mean plus two standard deviations (μ + 2σ). In total, 54 genotypes were selected: 27 from BB crosses, 17 from RR crosses, and 10 from collected elite lines.

Table 4. List of selected genotypes with high yield potential.

Entry	Designation	GD*	PH*	ET*	Yield*	Cluster	SES
G1	BR11716-4R-105	150	115	8	1.93	1	5
G2	BR11716-4R-120	161	148	9	1.92	1	5
G3	BR11712-4R-227	159	125	9	1.91	1	5
G4	BR11715-4R-186	159	113	8	1.90	1	5
G5	BR11712-4R-1	153	116	9	1.89	1	5
G6	**HRR271-4R-44**	139	138	8	1.89	2	3
G7	HRB510-4R-51	142	110	13	1.83	3	5
G8	**HRR271-4R-46**	139	147	11	1.83	2	3
G9	BR11723-4R-12	161	115	8	1.83	1	5
G10	BR11723-4R-27	160	117	8	1.83	1	5
G11	**HRB512-4R-108**	132	108	13	1.83	3	3
G12	HRB502-4R-64	137	126	14	1.82	3	5
G13	BBC12-4R-32	144	118	13	1.79	3	5
G14	**HRR269-4R-36**	130	133	11	1.79	2	3
G15	HRR272-4R-16	141	114	14	1.79	3	6
G16	HRR271-4R-45	138	142	11	1.77	2	5
G17	HRB502-4R-132	144	112	13	1.76	3	6

Entry	Designation	GD*	PH*	ET*	Yield*	Cluster	SES
G18	HRB502-4R-236	143	114	19	1.74	3	5
G19	HRB502-4R-35	141	117	16	1.74	3	6
G20	HRB511-4R-27	127	111	11	1.74	3	8
G21	BBC11-4R-35	141	103	11	1.72	3	5
G22	HRB502-4R-227	145	97	14	1.72	3	6
G23	**HRB513-4R-157**	142	117	12	1.72	3	3
G24	BBC14-4R-24	131	99	11	1.71	3	6
G25	**HRB512-4R-129**	136	103	13	1.71	3	3
G26	HRR269-4R-200	144	117	12	1.71	3	6
G27	BR11716-4R-108	161	115	7	1.71	1	3
G28	BBC14-4R-39	132	103	10	1.70	3	6
G29	HRR264-4R-91	129	134	17	1.70	3	7
G30	HRR271-4R-88	141	152	10	1.70	2	5
G31	HRB510-4R-202	134	101	11	1.69	3	8
G32	**HRB503-4R-4**	129	126	14	1.68	3	3
G33	HRR269-4R-15	135	128	9	1.68	3	5
G34	HRR269-4R-23	140	128	17	1.68	3	5
G35	HRR271-4R-63	140	142	15	1.68	3	5
G36	HRR264-4R-44	126	138	16	1.67	3	6
G37	HRR271-4R-32	139	160	10	1.67	2	6
G38	HRB502-4R-216	138	135	14	1.66	3	6
G39	BR11723-4R-172	159	118	7	1.65	1	5
G40	HRB502-4R-52	142	120	13	1.64	3	7
G41	RRC9-4R-51	145	112	12	1.60	3	8
G42	HRR264-4R-8	121	121	14	1.59	3	7
G43	HRB502-4R-68	135	120	10	1.62	3	6
G44	BBC14-4R-49	130	103	10	1.61	3	7
G45	**HRB512-4R-201**	143	114	11	1.64	3	3
G46	**BR11716-4R-102**	160	113	7	1.63	1	3
G47	HRB502-4R-223	135	117	16	1.56	3	6
G48	HRB511-4R-180	135	111	13	1.55	3	9
G49	HRB502-4R-40	139	112	12	1.54	3	6
G50	HRR269-4R-139	132	128	9	1.53	3	6
G51	HRB502-4R-181	127	110	14	1.52	3	6
G52	HRB502-4R-66	132	127	12	1.52	3	6
G53	BBC12-4R-95	139	112	12	1.51	3	5
G54	**RRC9-4R-31**	146	109	10	1.51	3	3
	Population means	136.14	115.16	11.35	1.19		
	SD	9.74	15.08	2.60	0.32		
	LSD (0.05)	9.27	8.92	4.70	0.53		
	Heritability (0.05)	0.90	0.96	0.75	0.80		

*GD: Growth duration (days), PH: Plant height (cm), ET: Effective tiller number, Yield: Rice grain yield in kg per 1.2 sq.m., SD: Standard deviation, LSD (0.05): Least significant difference at 5% level, Heritability (0.05): Heritability in broad sense at 5% level.

Diversity

Genetic distances among the selected 54 genotypes were measured using Euclidean distance. The highest genetic distance was observed between HRB502-4R-181 and BR11716-4R-120 (5.85), followed by HRR264-4R-8 and BR11716-4R-120 (5.56), HRB502-4R-223 and BR11716-4R-120 (5.27), BBC14-4R-49 and BR11716-4R-120 (5.22), HRR264-4R-8 and BR11715-4R-186 (5.14). The lowest genetic distance was observed in BR11723-4R-27 and BR11723-4R-12 (0.17) followed by HRB513-4R-157 and HRR269-4R-200 (0.22), HRB502-4R-40 and BBC12-4R-95 (0.26), BBC14-4R-24 and HRB510-4R-202 (0.37), BR11723-4R-172 and BR11716-4R-102 (0.41). Figure 3 illustrates the frequency distribution of genotype pairs based on pairwise Euclidean distances, which ranged from 0.17 to 5.85, indicating the degree of genetic divergence among the genotypes. The majority of genotype pairs exhibited moderate genetic dissimilarity, with the highest frequency (530 pairs) occurring at a distance class of >2-3. This is followed by 363 pairs at distance >3-4 and 337 pairs at distance >1-2, showing a roughly normal distribution centered around intermediate distances. In contrast, relatively few genotype pairs showed very low (distance 0-1; 61 pairs) or very high (distance 5-6; 11 pairs) divergence. A smaller number of pairs (129) were found at 4-5 distance class (Fig. 3).

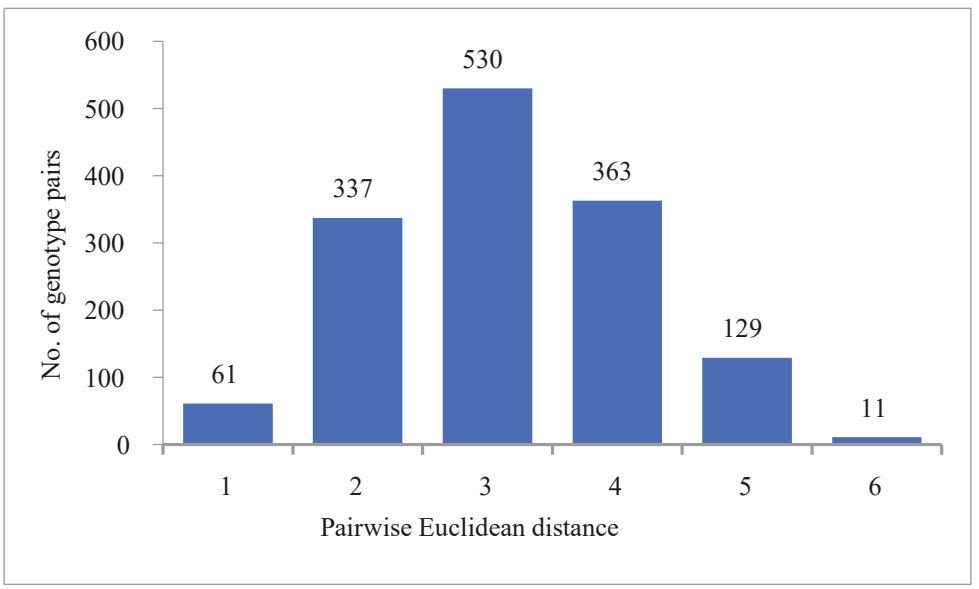


Fig. 3. Frequency distribution of genotype pairs based on pairwise Euclidean distance.

The 54 selected genotypes were then classified into three clusters using Wards D^2 method of clustering (Fig. 4). Clusters 1 and 2 contained potential restorer lines only, whereas cluster 3 contained both restorer and maintainer lines.

Cluster 1 comprised of late-maturing genotypes characterized by the longest growth duration (158.30 days), moderate plant height (119.50 cm), and a moderate number of effective tillers (8.00). Despite having only 10 genotypes, this cluster recorded the highest yield (1.82 kg/1.2 m^2). These results suggest that the late-maturing genotypes in this group were more productive, possibly due to their longer vegetative and reproductive phases, allowing for better assimilate accumulation and grain filling.

Cluster 2 consisted of medium-duration genotypes with an average growth duration of 137.67 days and the tallest plant height (145.33 cm). This group exhibited a moderately high

number of effective tillers (10.17) and achieved a yield of 1.78 kg/1.2 m². With only six genotypes, Cluster 2 represented a small group of tall, medium-duration types that performed moderately well in terms of yield, possibly combining traits of vigor and intermediate maturity (Fig. 4).

Cluster 3 included early-maturing genotypes with the shortest growth duration (136.68 days) and the shortest plant height (116.24 cm). Interestingly, this cluster had the highest number of effective tillers (12.89), yet it recorded the lowest yield (1.67 kg/1.2 m²). Containing 38 genotypes, it was the largest cluster, indicating that early-maturing, short-statured, and highly tillering genotypes were more common but less productive overall.

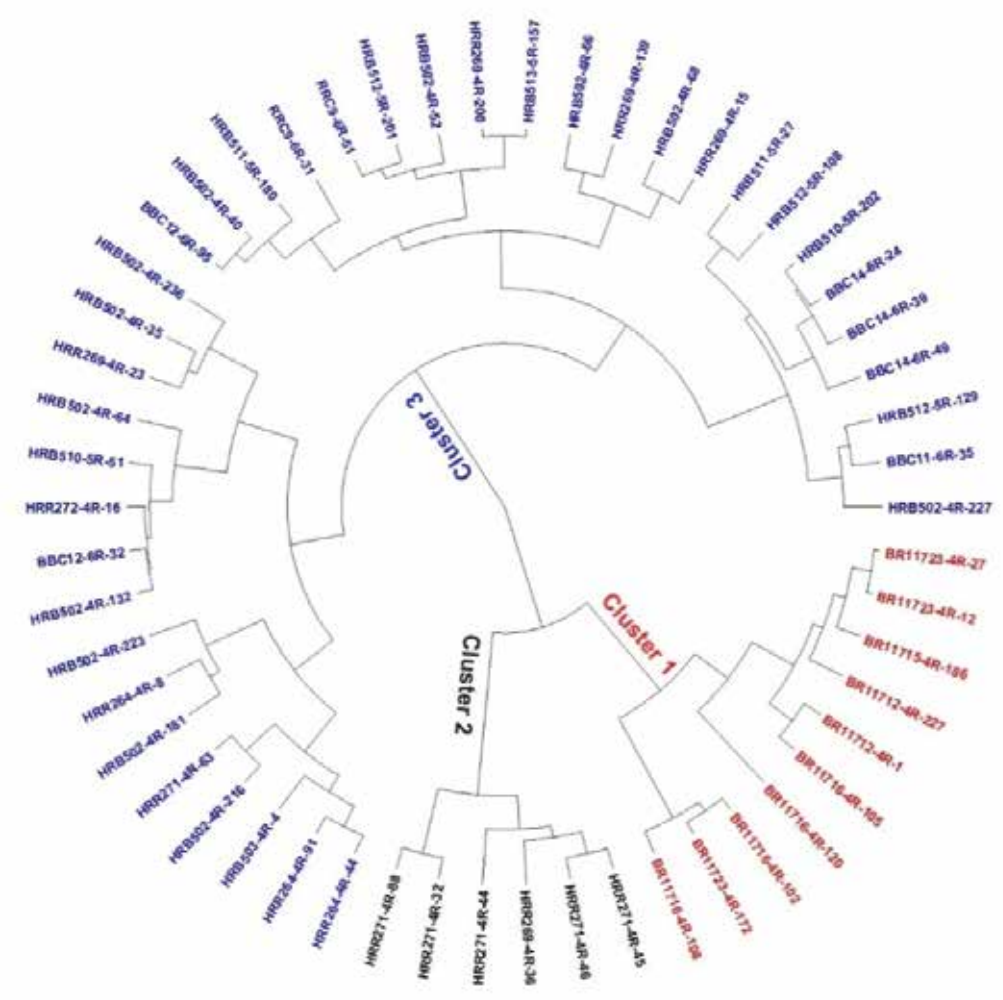


Fig. 4. Hierarchical Clustering using Ward's D² method

The intra-cluster distance table, represented by the average silhouette width values, provides an indication of how well each cluster is internally cohesive and distinct from other clusters. Cluster 1 exhibited the highest average silhouette width (0.464), followed closely by Cluster 2 (0.461), and cluster 3 (0.284).

Table 5. Intra and inter-cluster distances depicting the diversity among selected genotypes.

Distance	Intra-Cluster Distance	Inter-Cluster Distance		
	Average Silhouette Width	Euclidean Distance between Scaled Cluster Centers		
Cluster		1	2	3
1	0.464	0	2.88	3.07
2	0.461	2.88	0	2.48
3	0.284	3.07	2.48	0

The inter-cluster distances (Table 5), which shows Euclidean distances between the scaled cluster centers, reflects the degree of genetic divergence among the clusters. The greatest distance (3.07) was observed between Clusters 1 and 3, indicating the widest divergence between these two groups. The distance between Clusters 1 and 2 (2.88) was also substantial, while the smallest distance (2.48) occurred between Clusters 2 and 3, suggesting that these two clusters are relatively closer in multivariate space. Collectively, these results demonstrate that the clusters are reasonably distinct, with Clusters 1 and 3 being the most dissimilar, while Clusters 2 and 3 showing partial similarity or overlap.

Salinity tolerance of the selected lines

Fifty-four rice genotypes along with two susceptible checks (BRRI dhan28 and IR154) were evaluated using RCB design with two replications (Fig. 5). The standard evaluation score (SES) was utilized to select tolerant and susceptible genotypes. Among the selected 54 genotypes, six R lines (HRR271-4R-44, HRR271-4R-46, HRR269-4R-36, BR11716-4R-102, BR11716-4R-108, RRC9-4R-31) and five B lines (HRB512-4R-108, HRB513-4R-157, HRB512-4R-129, HRB503-4R-4, HRB512-4R-201) had SES score of 3 (tolerant). Nineteen genotypes had SES scores of 5 (moderately tolerant) and rest were susceptible having SES scores of 6-9 (Table 4).

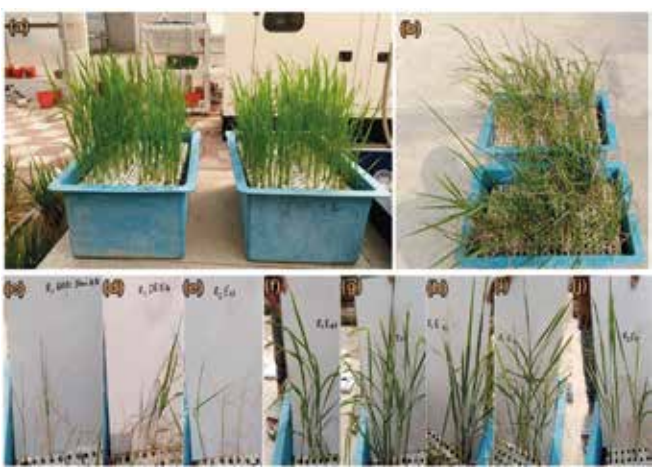


Fig. 5. Comparative Response of 56 Rice Genotypes (including two checks) to Salinity Stress (12 dS/m) at the Seedling Stage. (a) Seedling condition after one week of stress exposure. (b) Seedling condition after three weeks of stress exposure. (c) & (d) Appearance of susceptible checks BRRI dhan28 and IR154 during SES scoring. (e) Example of a susceptible genotype (HRB510-4R-202). (f-i) Examples of tolerant genotypes (HRB512-4R-201, HRR271-4R-44, BR11716-4R-102, and BR11716-4R-108). (j) Example of a moderately tolerant genotype (HRB510-4R-51) based on SES scoring.

Confirmation of Restoring and Maintaining Ability

To identify the restorer and non-restorer alleles of the fertility restorer genes Rf3 and Rf4, the DRRM-Rf3-5 and DRCG-RF4-14 gene-based markers were used, respectively (Table 3, Fig. 6).

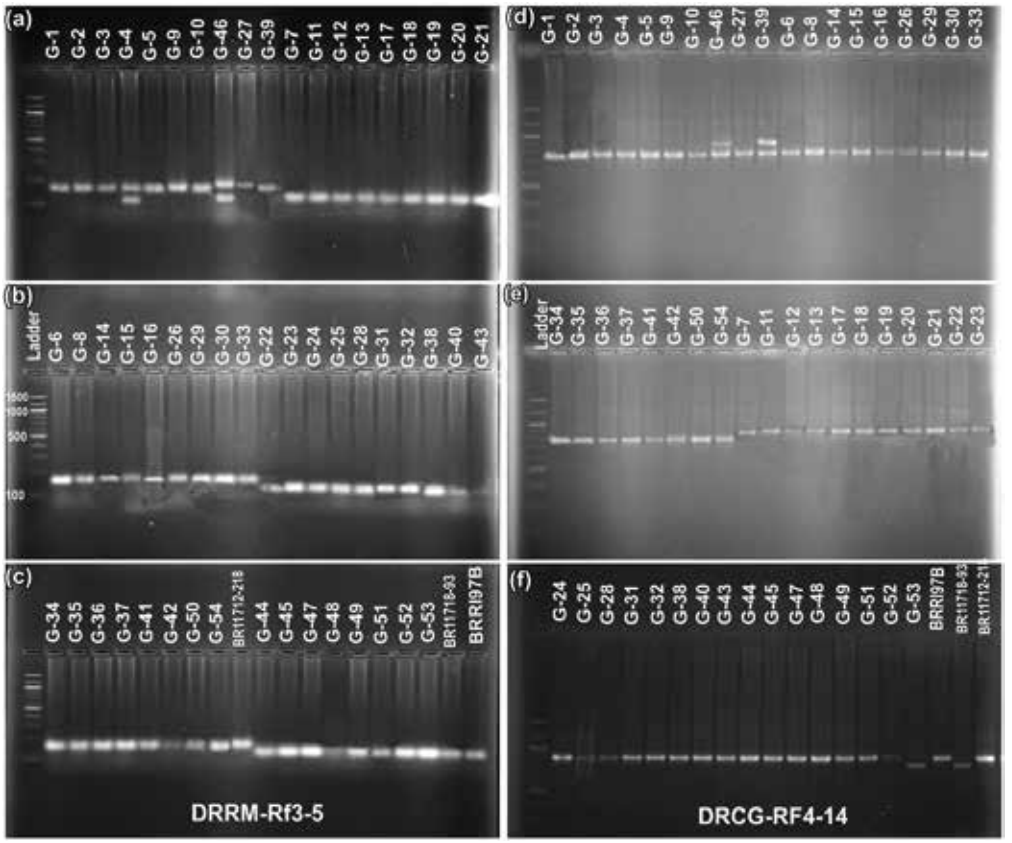


Fig. 6. Marker-aided selection for *Rf3* and *Rf4* genes. (a,b,c): DRRM-Rf3-5 marker showing characteristic bands for G1-G54, BR11712-4R-218, BR11718-4R-93 and BRRI97B (B line for wild abortive cytoplasm of BRRI97A). (d,e,f): DRCG-RF4-14 marker showing characteristic bands for G1-G54, BR11712-4R-218, BR11718-4R-93 and BRRI97B.

The restorer (R line) genotypes showed a homozygous band at 160 bp with the DRRM-Rf3-5 marker and an 800 bp band with the DRCG-RF4-14 marker. In contrast, the maintainer (B line) genotypes produced a homozygous band at 140 bp for the DRRM-Rf3-5 marker and a 885 bp band for the DRCG-RF4-14 marker. Using these markers, 24 genotypes were confirmed as restorer line (R line) and 27 lines as maintainer lines (B line) (Fig. 6). Three selected elite lines BR11715-4R-186 (G4), BR11723-4R-172 (G39), BR11716-4R-102 (G46); and two unselected elite lines BR11712-4R-218, BR11718-4R-93 were discarded due to heterozygosity in the *Rf3* and/or *Rf4* locus.

Pollen Fertility Assessment in test cross F1 Plants

Pollens produced three different staining categories: completely transparent (no stain absorbed), mixture of transparent and black colored pollens and >80% black pollens (Fig. 7). Completely transparent pollens were regarded as sterile, whereas, black colored pollens were classified as fertile.

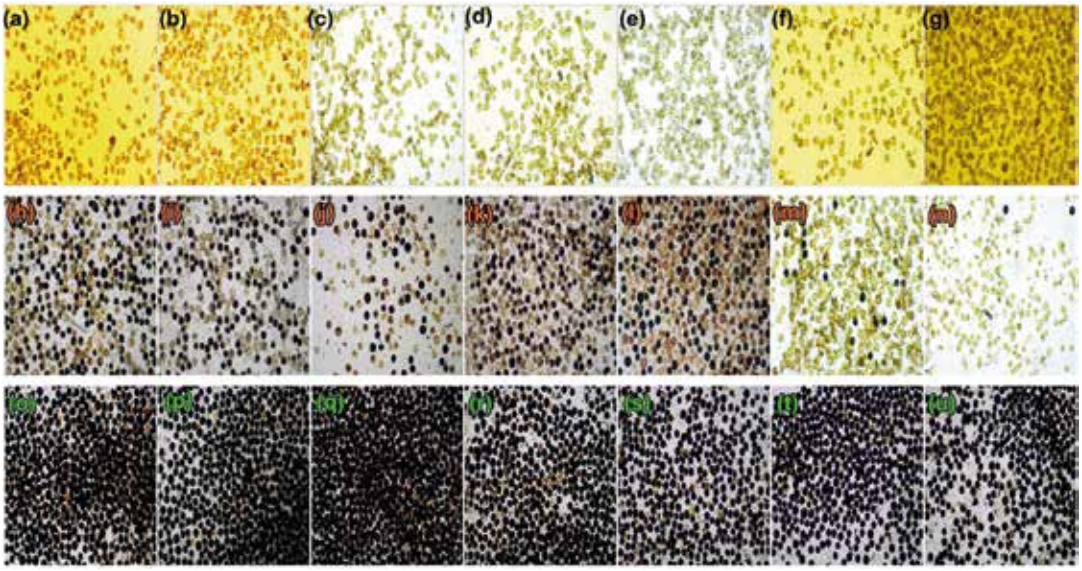


Fig. 7. Pollen test at test cross F_1 progeny (BRRI97A×Elite lines) for Confirmation of restoring and maintaining ability. (a-g): Pollen view of selected new maintainer lines. (h-l): Pollen view of discarded lines due to <80% fertility (BR11715-4R-186 (G4), BR11723-4R-172 (G39), BR11716-4R-102 (G46), BR11712-4R-218, BR11718-4R-93). (m,n): Pollen view of discarded lines due to incomplete sterility of BBC14-4R-49 (G44) and HRB502-4R-181 (G51). (o-u): Pollen view of selected new restorer lines.

Test cross F_1's from the B×B cross derived selected 27 fixed lines, 25 produced 100% sterile pollen (Fig. 7: a-g) and confirmed as new maintainer lines, but two genotypes produced 90-98% sterile pollens (Fig. 7: m,n) and were discarded. Test cross F_1's from the R×R cross derived selected 17 fixed lines produced >80% fertile pollens and selected as new R lines. Test cross F1's from the tested 12 elite lines, 7 produced >80% fertile pollen and identified as new R line, whereas 5 produced <80% fertile pollen and were discarded (Fig. 7: h-l). Though BBC14-4R-49 (G44) and HRB502-4R-181 (G51) showed recessive band for both the used markers but they could not show 100% sterility and showed 90-98% sterility (Fig. 7: m-n).

DISCUSSION

The development of salinity-tolerant hybrid rice parental lines is critical for sustaining rice production in salt-affected areas, which are expanding due to climate change, sea-level rise, and poor irrigation management (Raghavendra *et al.*, 2021). In the present study, 286 genetically fixed entries derived from 22 crosses (14 B×B and 8 R×R) and 19 elite lines were evaluated for salinity tolerance using the Standard Evaluation System (SES) and agronomic performance. The strategic approach of crossing parents with favorable SES scores (3-5) aimed to combine high salinity tolerance with desirable grain yield with agronomic traits and fertility restoration/maintenance ability in the progenies.

Parental Selection Strategy and Cross Performance

The identification of 15 promising crosses (9 B×B and 6 R×R), where both parents exhibited SES scores of 3-5 represents a focused breeding strategy for introgressing salinity tolerance into new hybrid rice parents. This approach aligns with recent marker-assisted breeding programs that have successfully combined salinity tolerance with other desirable traits in parental lines (Niu *et al.*, 2025; Sheng *et al.*, 2023). Among these, cross HRB 502 (BHR101/BHR184) produced the largest F_6

population (33 entries). The varying parental SES scores and F_6 population sizes (ranging from 1 to 33) across the crosses provide a diverse genetic pool for selecting high-yielding and salinity-tolerant fixed lines for use as new hybrid rice parents. The use of tolerant × tolerant crosses is supported by previous studies showing that parental salinity tolerance is a prerequisite for developing stable salt-tolerant hybrids (Raghavendra *et al.*, 2021; Beulah *et al.*, 2023).

The wide variation in F_6 population sizes (1-33 entries) across crosses reflects differences in cross compatibility, seed set, and selection pressure applied during line stage testing (LST) trial. The seven crosses involving at least one highly susceptible parent (SES 7-8) yielded fewer promising selections, confirming that both parents should possess moderate to high tolerance for efficient introgression of this trait. Similar observations have been reported in studies where screening of large germplasm collections identified maintainer and restorer candidates with inherent salinity tolerance for hybrid development (Beulah *et al.*, 2023; Dolo *et al.*, 2016).

BLUP-Based Selection for High-Yielding Genotypes

The application of Best Linear Unbiased Predictor (BLUP) values for selection based on yield, growth duration, plant height, and effective tiller number represents a robust statistical approach for handling the augmented randomized complete block design with unbalanced data. BLUP-based selection has gained prominence in plant breeding due to its ability to account for spatial variation and provide unbiased estimates of genotypic values (Piepho *et al.*, 2008). In this study, the stringent selection threshold of population means plus one standard deviation ($\mu + 1\sigma$) for yield resulted in 54 selected genotypes, while only five exceptional genotypes exceeded $\mu + 2\sigma$. This selection intensity ensures that only superior performers are advanced, maximizing genetic gain while maintaining sufficient genetic diversity for future breeding efforts (Falconer and Mackay, 1996).

The distribution of selected genotypes across breeding populations (27 from B×B crosses, 17 from R×R crosses, and 10 from elite lines) indicates successful recovery of high-performing lines from diverse genetic backgrounds. The elite lines contributed proportionally fewer selections, suggesting that the targeted crosses were more effective in generating superior combinations. This outcome validates the crossing strategy and emphasizes the importance of planned hybridization over relying solely on existing germplasm.

Genetic Diversity and Cluster Analysis

The Euclidean distance analysis revealed substantial genetic diversity among the 54 selected genotypes, with distances ranging from 0.17 to 5.85. The highest genetic distance between HRB502-4R-181 and BR11716-4R-120 (5.85) indicates these lines are highly divergent and could serve as complementary parents in future hybrid combinations to maximize heterosis (Sruthi *et al.*, 2020; Prasanna *et al.*, 2022). Conversely, the lowest distance between BR11723-4R-27 and BR11723-4R-12 (0.17) suggests these lines are closely related, likely derived from the same cross with minimal phenotypic differentiation.

The frequency distribution of pairwise distances showed a roughly normal distribution centered around moderate distances (2-4 units), with the highest frequency (530 pairs) at distance >2-3. This pattern indicates a balanced level of genetic variability within the selected population, which is desirable for maintaining breeding flexibility while ensuring sufficient divergence for heterotic exploitation (Prasanna *et al.*, 2022). The relatively few pairs showing very low (0-1; 61 pairs) or very high (5-6; 11 pairs) divergence further supports the notion that the population represents a well-structured gene pool with moderate diversity.

Clustering Patterns and Agronomic Implications

Ward's D2 hierarchical clustering classified the 54 genotypes into three distinct clusters with

contrasting agronomic characteristics. Cluster 1, comprising late-maturing genotypes (158.30 days), exhibited the highest yield (1.82 kg/1.2 m²) despite having only 10 members and moderate tiller numbers (8.00). This suggests that extended growth duration allows for greater biomass accumulation and enhanced grain filling, consistent with reports that late-maturing varieties often achieve higher yields through prolonged photosynthetic activity and better resource utilization (Yang *et al.*, 2010). These genotypes are particularly valuable for developing high-yielding hybrids in environments where the growing season permits late maturity.

Cluster 2 consisted of medium-duration genotypes (137.67 days) characterized by the tallest plant height (145.33 cm) and moderate yield (1.78 kg/1.2 m²). Tall plant stature in hybrid rice parents can be advantageous for biomass production and lodging resistance when combined with appropriate stem strength traits (Khush *et al.*, 2005). However, excessive height may increase lodging risk under high-input management, necessitating careful evaluation in hybrid combinations.

Cluster 3, the largest group with 38 members, included early-maturing, short-statured genotypes (136.68 days, 116.24 cm) with the highest tiller number (12.89) but the lowest yield (1.67 kg/1.2 m²). This inverse relationship between tiller number and yield suggests a trade-off where high tillering may result in smaller panicles or reduced grain weight per panicle, possibly due to competition for assimilates among tillers (Huang *et al.*, 2011). Previous studies have demonstrated that while effective tiller number is positively correlated with yield, excessive tillering can lead to unproductive tillers and reduced individual panicle productivity (Huang *et al.*, 2011). This cluster represents genotypes suitable for breeding programs targeting early maturity and adaptation to short-season environments, though yield improvement would require selection for larger panicle size or grain weight.

Cluster Validation and Inter-Cluster Divergence

The silhouette width analysis confirmed the clustering structure, with Clusters 1 and 2 exhibiting higher average silhouette widths (0.464 and 0.461, respectively) compared to Cluster 3 (0.284). High silhouette values indicate that genotypes within these clusters are well-grouped and distinct from other clusters, reflecting strong internal cohesion (Rousseeuw, 1987). The lower silhouette width of Cluster 3 suggests greater internal variability and potential overlap with other clusters, which may be attributed to its larger size and broader genetic composition.

The inter-cluster distance analysis revealed that Clusters 1 and 3 were the most divergent (distance 3.07), indicating substantial phenotypic differentiation between late-maturing, high-yielding types and early-maturing, high-tillering types. This divergence provides opportunities for heterotic crosses between these groups to exploit complementary traits (Sruthi *et al*., 2020). The smallest inter-cluster distance between Clusters 2 and 3 (2.48) suggests some phenotypic similarity, possibly in maturity duration, which could limit heterotic potential in crosses between these groups.

Salinity Tolerance Evaluation of Selected Lines

The evaluation of 54 selected genotypes under 12 dS/m salinity stress at the seedling stage identified 11 highly tolerant lines (SES 3): six restorer lines and five maintainer lines. This represents a 20.4% success rate in recovering highly tolerant lines from the selected population, which is consistent with the expected segregation patterns when crossing moderately tolerant parents (Beulah *et al.*, 2023; Singh and Flowers, 2010). An additional 19 genotypes exhibited moderate tolerance (SES 5), providing a broader pool of genetic material for further improvement through recurrent selection or marker-assisted backcrossing.

The visual differences in seedling response to salinity stress (Fig. 5) clearly distinguished

tolerant genotypes (minimal leaf damage, continued growth) from susceptible ones (severe leaf scorching, stunted growth), validating the SES scoring system as an effective phenotyping tool. The susceptible checks BRRI dhan28 and IR154 displayed severe stress symptoms, confirming the effectiveness of the screening protocol. The SES-based screening approach has been widely used in rice breeding programs and has proven reliable for identifying salinity-tolerant donors and breeding lines (Raghavendra *et al.*, 2021; Beulah *et al.*, 2023; Dolo *et al.*, 2016).

The recovery of both highly tolerant and moderately tolerant lines from the breeding populations confirms the effectiveness of the crossing strategy. The tolerant lines identified in this study can serve as new parental lines for hybrid rice breeding programs targeting salt-affected ecologies, while moderately tolerant lines can be used as donors in marker-assisted backcrossing programs to further enhance salinity tolerance (Niu *et al.*, 2025; Thomson *et al.*, 2010).

Molecular Confirmation of Fertility Restoration and Maintenance Ability

The use of gene-based markers DRRM-*Rf3*-5 (for *Rf3*) and DRCG-RF4-14 (for *Rf4*) successfully differentiated restorer lines from maintainer lines at the molecular level. The *Rf3* gene on chromosome 1 and *Rf4* gene on chromosome 10 are the major fertility restorer genes in rice, encoding pentatricopeptide repeat (PPR) proteins that restore pollen fertility in cytoplasmic male sterile (CMS) lines (Huang *et al.*, 2015). The clear banding patterns observed-160 bp and 800 bp for restorer lines versus 140 bp and 885 bp for maintainer lines-enabled efficient classification of 24 genotypes as restorers and 27 as maintainers.

Molecular markers for *Rf* genes have been extensively validated and are routinely used in hybrid rice breeding programs to accelerate parental line development (Huang *et al.*, 2015; Kumar *et al.*, 2017; Tang *et al.*, 2014). Studies have reported marker efficiencies of 80-90% for SSR and gene-based markers linked to *Rf3* and *Rf4*, making them valuable tools for preliminary screening of breeding materials (Kumar *et al.*, 2017; Sheeba *et al.*, 2009). However, the efficiency of these markers is not 100%, necessitating phenotypic confirmation through test cross evaluation (Tang *et al.*, 2014; Nagaraju *et al.*, 2023).

Pollen Fertility Testing and Phenotypic Validation

The test cross evaluation using I-KI staining provided phenotypic confirmation of fertility restoration and maintenance ability. Among the 27 B×B-derived lines tested, 25 produced 100% sterile pollen in test cross F1s, confirming their maintainer status, while two genotypes (BBC14-4R-49 and HRB502-4R-181) showed only 90-98% sterility and were discarded. This discordance between molecular marker prediction and phenotypic expression highlights the limitation of relying solely on molecular markers for parental line classification (Tang *et al.*, 2014; Nagaraju *et al.*, 2023).

Several factors may contribute to incomplete sterility in putative maintainer lines despite showing recessive alleles for both *Rf3* and *Rf4*. These include: (1) environmental effects on pollen fertility expression (Li and Yang, 2007); (2) allelic variation at the *Rf* loci that affects restoration efficiency (Huang *et al.*, 2009); and (3) possible errors in marker scoring or DNA quality issues. Similar observations have been reported in other studies where marker-based predictions did not perfectly align with phenotypic fertility restoration, emphasizing the need for combined molecular and phenotypic selection (Tang *et al.*, 2014; Nagaraju *et al.*, 2023; Kumar *et al.*, 2016).

All 17 R×R-derived lines and 7 out of 12 tested elite lines produced >80% fertile pollen in test cross F1s, confirming their restorer status. The threshold of >80% pollen fertility is commonly used in hybrid rice breeding to classify effective restorers when both *Rf3* and *Rf4* are present in homozygous dominant condition (Kumar *et al.*, 2017). The five elite lines that failed to meet this threshold were appropriately discarded, demonstrating the value of phenotypic

validation in eliminating false positives from molecular screening. Among the selected 54 genotypes, six R lines (HRR271-4R-44, HRR271-4R-46, HRR269-4R-36, BR11716-4R-102, BR11716-4R-108, RRC9-4R-31) have SES 3 score (tolerant) and confirmed as new restorer lines except BR11716-4R-102. Five B lines HRB512-4R-108, HRB513-4R-157, HRB512-4R-129, HRB503-4R-4, HRB512-4R-201 have SES 3 score (tolerant) and confirmed as new maintainer (B) lines.

Implications for Hybrid Rice Breeding

The successful development of 25 new maintainer lines (5 maintainer having SES 3) and 24 new restorer lines (5 restorer having SES 3) with combined salinity tolerance and desirable agronomic traits represents a significant achievement for hybrid rice breeding programs targeting salt-affected areas. These parental lines can be immediately utilized in hybrid combination trials to develop salt-tolerant hybrids with improved yield potential. The diversity analysis revealed sufficient genetic divergence among the selected lines to enable heterotic grouping and systematic hybrid development.

The integration of phenotypic screening (SES scoring, yield evaluation), molecular marker analysis (*Rf* gene markers), and statistical genetics (BLUP, clustering) in this study exemplifies a comprehensive and efficient approach to parental line development. This multi-faceted strategy maximizes selection efficiency while ensuring that only lines with confirmed genetic and phenotypic attributes are advanced (Niu *et al.*, 2025; Collard and Mackill, 2008). Future research work should focus on: (1) evaluating the combining ability of these parental lines through line × tester analysis; (2) assessing salinity tolerance at reproductive stage in addition to seedling stage; (3) incorporating additional trait-linked molecular markers for other important traits such as grain quality, disease resistance, and yield-related QTLs; and (4) conducting multi-location trials to assess stability and adaptability of salinity tolerance and yield performance across diverse saline environments.

CONCLUSION

This study successfully developed 49 new salinity-tolerant hybrid rice parental lines (25 maintainers and 24 restorers) through strategic crossing of tolerant donor parents, BLUP-based selection, and combined molecular-phenotypic validation. The newly developed parental lines need to be genotyped to identify which mechanisms and underlying genes/QTLs introgressed into the developed lines and provided tolerances. The identified lines exhibited diverse agronomic characteristics, grouped into three distinct clusters representing different growth duration-yield contributing ideotypes. The integration of SES-based salinity screening with molecular marker analysis for Rf genes proved effective, though phenotypic validation through test cross pollen fertility testing is crucial for confirming parental line classification. Overall, these newly developed parental lines provide valuable genetic resources for breeding salinity-tolerant hybrid rice varieties suited to salt-affected ecologies.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY STATEMENT

The data are available from the corresponding authors upon reasonable request.

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